Appendix E

Lab Studies White Papers

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Evaluating Differences in the Microbial Community Structure of Alternate Electron Donor Enrichments Using Molecular Methods

1. INTRODUCTION AND BACKGROUND

1.1 Test Area North In Situ Bioremediation

The aquifer at Test Area North (TAN) at the Idaho National Engineering and Environmental Laboratory (INEEL) is currently undergoing enhanced in situ bioremediation (ISB) to treat the residual source area of a large contaminant plume of chlorinated solvents including trichloroethene (TCE). This bioremediation is achieved by injection of an electron donor (sodium lactate), which provides a carbon and energy source for the microbial community, thereby facilitating a redox reduction in the aquifer and driving the anaerobic reduction of TCE to ethene. The injection of electron donor is necessary to promote the growth and metabolism of a microbial community large enough to achieve appreciable rates of TCE reduction. Although lactate works well, other electron donors may potentially work better and/or cost less to use. In evaluating potential alternate electron donors (AEDs), several key parameters must be assessed, including but not limited to:

- The effect of the AED on the microbial community structure, specifically whether or not the AED selects for microbes responsible for the anaerobic reduction of TCE
- How different the AED microbial community is compared with other donors (lactate), and how difficult would it be to restore the original microbial community if it is decided to switch from an AED back to the original donor.

The effect of the AED on microbial community structure, specifically what portion of the community it is selected for, can be determined by using molecular methodologies. Molecular methods use DNA extracted from samples to determine their microbial community fingerprint. They can subsequently be used to assess qualitative or semi-quantitative differences in the community composition over time and/or under a change in stimulus, and can also look for a specific organism (or group of organisms) of interest. These methods can be used to provide a quick and relatively easy assessment of the effect of the AED on the microbial community of interest.

Enrichment cultures derived from TAN groundwater were developed in the laboratory. These cultures were enriched using the following electron donors:

- Whey
- Food-grade molasses
- Feed-grade molasses
- Sodium lactate.

All other conditions, including TCE concentrations, samplings, amendments of donor, and temperature, were maintained identical. Two years after these enrichments were developed, a history of TCE reduction performance was established. Significant differences were observed and it was hypothesized that these differences may be attributed to differences in the microbial community structure. Thus, the molecular analyses terminal restriction fragment length polymorphism (T-RFLP) and polymerase chain reaction (PCR) (specific for *Dehalococcoides ethenogenes*) were performed to determine the differences in species composition between the enrichments.

1.2 Background

The two molecular methods used (PCR and T-RFLP) focused on the 16S rRNA gene. This gene codes for the RNA portion of the small subunit of the bacterial ribosome, which is used to make proteins in microbial biosynthesis. Since all bacteria make protein, all bacteria have this gene, which makes it very useful in evaluating the composition of a microbial community. Differences between microbes are inferred by differences in the DNA base sequence (as adenine, thymine, guanine, and cytosine) of their 16S rDNA genes. Tentative identification of microbes within a community can also be achieved by comparing the similarity of the 16S rDNA sequences of those microbes with sequences compiled in large databases of all other known *Bacteria* and *Archaea*. Thus, identification of species within the community may be determined using these molecular methods.

Polymerase chain reaction is the process of amplifying a specific region of DNA within the microbial genome. It mimics the natural process of DNA replication using primers to target the regions to be amplified. For the analyses performed here, the specificity of the amplification was directed by targeting different portions of the 16S rRNA gene. Thus, whole populations or specific species were amplified and analyzed.

Terminal restriction fragment length polymorphism is a fingerprinting technique in which a community's PCR-amplified 16S rDNA gene sequences, with each microbe represented by a unique 16S rDNA sequence, are separated after digestion of the DNA with restriction endonucleases. Digestion results in the fragmentation of different 16S rDNA sequences into different sizes. The digested DNA is run on a DNA sequencer, which separates the DNA terminal restriction fragments (T-RFs) in the sample according to differences in length, and the intensity of each T-RF is assessed relative to an internal standard using software. The resulting chromatogram provides a community diversity profile illustrating the total number of the T-RFs and their peak heights (intensity). The chromatogram can be used to infer the species richness of a community by assuming that each T-RF represents a different species, and also infers the relative abundance of each species by quantifying the differences in peak height for each T-RF.

2. MATERIALS AND METHODS

Samples were collected from each of the four AED enrichment cultures. The DNA was extracted from these samples, with PCR and T-RFLP analyses performed to obtain a microbial community fingerprint of each to determine what differences existed between them and to assess whether or not *Dehalococcoides ethenogenes* rDNA could be detected. This section describes the materials and methods used to perform these analyses.

2.1 Alternate Electron Donor Culture Development and Amendments

A laboratory study was conducted to evaluate the potential for using electron donors other than sodium lactate to stimulate biodegradation of chlorinated ethenes in the TAN hot spot. Bioreactors were initially set up with sterile basalt, groundwater from TAN-25, and sodium lactate as an electron donor and spiked with TCE. Once consistent dechlorination was observed, the culture from the best-performing bioreactor was used to inoculate several flasks that were given AEDs. The electron donors evaluated were feed-grade molasses, food-grade molasses, cheese whey, and sodium lactate (the positive control) (see Table 1). The results of the study indicated that sodium lactate produced the most efficient and complete dechlorination to ethene, but that whey and food-grade molasses also performed well. The feed-grade molasses culture did not degrade all parent compound within the timeframe of the study. Details of this study are reported in the *Fiscal Year 2002 Alternate Electron Donor Evaluation, Test Area North Final Remedy, Operable Unit 1-07B* (INEEL 2002). Samples were collected to evaluate the impact of the AEDs on the composition of the microbial community, and those results are reported in this document.

Table 1. Concentrations of TCE and electron donor added at each amendment to the cultures.

Flask number	Electron donor	TCE (mg/L)	Electron donor added as chemical oxygen demand (COD) (g)
1	Food-grade Molasses	10	0.013
2	Feed-grade Molasses	10	0.013
3	Cheese Whey	10	0.013
4	Sodium Lactate	10	0.008
5	(Abiotic Control) Sodium Lactate	10	0.008

2.2 Sample Collection and DNA Extraction

Samples were collected (10 mL) from each of the AED enrichment cultures. A rigorous sterile technique was used during all procedures throughout the sampling and analyses. The samples were centrifuged at 20,000 rpm for 20 minutes to obtain a cell pellet. The supernatant was decanted and the pellet was re-suspended in Dnase-free water (500 µL). The pellet was then stored at -80°C until DNA extraction was performed. The DNA extraction was performed using the *Fast DNA Spin Kit for Soil* (Quiagen Inc., Valencia CA) according to the manufacturer's instruction. The extracted DNA was stored at -20°C until further analyses were performed.

2.3 Polymerase Chain Reaction

Polymerase chain reaction was performed to exponentially amplify the 16S rDNA portion of the microbial genome of all the microbes in the samples. Primers were used to direct the amplification in three ways: (1) universal bacterial primers were used to amplify all *Bacteria* rDNA within each sample, (2) universal archaeal primers were used to amplify all *Archaea* rDNA within each sample, and (3) primers specific for *Dehalococcoides ethenogenes* were used to attempt to amplify this specific organism's rDNA from each sample. The PCR reactions were performed in a sterile laminar flow hood, which was used exclusively for PCR. The hood was washed with 70% ethanol and 10% bleach and ultraviolet (UV)-irradiated for 10 minutes before each setup. All reagents used were certified Dnase/Rnase free. The standard PCR reaction included 0.4 mg/mL BSA, 1X PCR buffer (Promega, Madison WI), 1.5 mM magnesium chloride, 0.5 µM forward and reverse primer (Invitrogen, Carlsbad CA), 1U Taq polymerase (Promega), 0.2 µM dNTPs (Gibco, Carlsbad CA), and 1 µL of extracted DNA. DNA amplification was performed on a model 9600 thermal cycler (Perkin Elmer Inc. Wessley, MA). The PCR products (5µL + 1µL 6X loading dye [Promega]) were electrophoresed on a 1.0-1.2% (wt/vol) agarose gel in 1X TBE buffer containing of Ethidium Bromide (1 µg/mL) at 70V for 30 minutes. The gels were photographed with an Alpha Imager and software (Alpha Innotech, San Leandro, CA).

2.4 Terminal Restriction Fragment Length Polymorphism

Terminal restriction fragment length polymorphism was performed on PCR-amplified *Bacteria* and *Archaea* rDNA from each sample. The PCR was performed per Section 2.2, except the forward primer was labeled with FAM (phosphoramidite fluorochrome 5-carboxyfluorescein). PCR was performed in triplicate for each sample, the products were combined (150 µL total combined volume), and then they were purified using the Quiaquick PCR cleanup kit (Quiagen Inc.) according to the manufacturer's instructions. The purified PCR products were quantified by reading a 1:100 dilution of the products in a UV Spectrophotometer at 260 nm. According to the manufacturer's instruction, 200 ng of purified PCR product was digested with *MspI* (*Bacteria*) or *HhaI* (*Archaea*). The digestion reactions were purified by ethanol precipitation. Samples were then prepared for loading onto a 377 DNA sequencer (ABI) by heat shocking the DNA digests in a 95°C water bath for exactly 3 minutes and then transferring the digested DNA to an ice bath to cool. The samples were then centrifuged for 1 minute to denature the DNA. The denatured DNA (2 µL) was then loaded onto the DNA sequencer. The samples were analyzed along with the internal standard Rox 1000 (ABI) and the resulting sequence information was analyzed using Genescan (ABI) software.

2.5 Normalization and Statistical Analysis of Terminal Restriction Fragment Length Polymorphism Profiles

The assumption for diversity calculations was that each T-RF within a community profile represented a unique species within the community. The diversity of the AED culture samples was assessed after normalization of the triplicate T-RFLP profiles was performed. The triplicate profiles were aligned manually, and T-RFs in different replicates that differed by less than 0.5 bp were considered identical and were clustered. All T-RFs within a cluster were assigned the average of the base pair size associated with T-RFs within the cluster. The peak heights of the clustered T-RFs were also averaged. In order to ensure that diversity analyses were performed on profiles that were rigorously produced, any T-RF not occurring in all of the replicate profiles was discarded. Also, any T-RF within the clustered profile with peak height averaging ≤ 25 florescence units for *Bacteria* and ≤ 50 florescence units for *Archaea* were discarded. These composite profiles were used to determine the species richness and evenness parameters. The sum of all peaks in each derivative bacterial and archaeal profile was calculated as an indication of the total DNA quantity represented by the profile.

In order to compare the composite profiles of different samples, the DNA quantity was standardized between the different sample profiles to the smallest quantity by proportionally reducing the peak height of each T-RF in the larger profiles. The proportion of the smallest DNA quantity and the larger DNA quantity was calculated and used as a correction factor to adjust each peak height in the profile with the larger DNA quantity. Thus, the total adjusted DNA of each profile was the same so that the relative abundance of T-RFs within the profiles could be calculated.

Once the community profiles were normalized, statistical parameters to measure diversity, relative abundance, and similarity were calculated so that the communities could be compared. The Jaccard coefficient (Dunbar 2001) was used to estimate similarity of the communities. A matrix was created that indicated the presence or absence of a particular T-RF in all the composite profiles being compared, and the matrix was input into the Estimates® software. The Jaccard coefficient was used because it describes the similarity of each sample pair based only on the T-RFs that are present in one or both samples. Thus, the T-RFs not present in either of the two samples being compared do not contribute to the similarity of the two samples. This is especially useful when comparing multiple profiles that are very diverse. The Jaccard coefficient is calculated by:

$$S = \frac{\sum S_s}{\sum S_T}$$

where

S = Jaccard coefficient

S_s = shared T-RFs between samples

S_T = T-RFs in samples.

Each T-RF within the T-RFLP profile was assumed to represent one species within the microbial community. The statistical parameters used to assess diversity require this assumption, although it is not necessarily true and one fragment may actually represent multiple species within the microbial community. The diversity of fragments within the community profiles (i.e., richness and evenness) was determined by the Shannon-Weiner Diversity Index and the Simpson Diversity Index (Dunbar 2000). The formula used to calculate the Shannon-Weiner Diversity Index was:

$$H' = -\left[\sum (p_i)(\ln(p_i))\right]$$

where

H' = diversity

p_i = $\frac{fu_{T-RF}}{\sum fu_{T-RF}}$, proportion of each individual fragment fluorescence (fu) to the total.

This parameter evaluates the total number of species (T-RFs) and their relative abundance within the community (evenness). Communities that have a high number of species, but that contain a very predominant species, will have a lower Shannon-Weiner diversity number than a community with a low number of species where the species are all similar in proportion.

The other parameter used to assess diversity of the T-RFLP profiles was the Simpson Diversity Index. The formula used was:

$$D = \frac{1}{\sum (p_i^2)}$$

where

D = diversity

p_i = $\frac{fu_{T-RF}}{\sum fu_{T-RF}}$, proportion of each individual fragment fluorescence (fu) to the total.

This parameter is similar to the Shannon-Weiner Diversity index, and both were used to corroborate the representative values calculated for each.

3. RESULTS AND DISCUSSION

This section presents the microbial community analyses of the four AED laboratory cultures. It discusses the results of the T-RFLP analyses (which included a visual interpretation of the chromatograms), evaluation of the statistical parameters measured, and detection of *Dehalococcoides ethenogenes*.

3.1 Detection of Dehalococcoides Ethenogenes

Amplification of *Dehalococcoides ethenogenes* rDNA, using PCR primers specific for this bacteria, was achieved in all of the AED cultures. This analysis was performed for detection only; quantification of the PCR products was not done.

3.2 Terminal Restriction Fragment Length Polymorphism: *Bacteria*

The number of bacterial species detected by T-RFLP for each AED culture, and hence the species similarity (Jaccard coefficient [Dunbar 2001]), varied (see Table 2). Thirty-one species were detected in the food-grade molasses culture, 22 species were detected in the feed-grade molasses culture, 21 species were detected in the cheese whey culture, and 40 species were detected in the sodium lactate culture. According to the Jaccard coefficient of similarity, the feed- and food-grade molasses cultures shared the greatest percentage of species (0.56), which was not surprising given the similarity in the electron donor source. The feed-grade molasses and sodium lactate cultures (0.35), and cheese whey and sodium lactate cultures (0.33) were the least similar (see Table 2). This makes sense given the feed-grade molasses and cheese whey were not pure electron donors and may have introduced other bacterial species into the cultures. The food-grade molasses was the most similar to the sodium lactate culture (0.45), which may have been due to the purity of the substrates. Table 2 illustrates the total number of species detected within the T-RFLP profile of each culture, the total number of species that were shared between two cultures, and the Jaccard coefficient of similarity calculated for each. Overall, these data suggest that all of the cultures were significantly different, with similarity values ranging from 0.33 to 0.56.

Table 2. Species similarity of alternate electron donor cultures.

AED	AED	Species Detected (AED)	Species Detected (AED)	Shared Species	Jaccard Coefficient
Food-grade molasses	Feed-grade molasses	31	22	19	0.56
Food-grade molasses	Cheese whey	31	21	14	0.37
Food-grade molasses	Sodium lactate	31	40	22	0.45
Feed-grade molasses	Cheese whey	22	21	15	0.54
Feed-grade molasses	Sodium lactate	22	40	16	0.35
Cheese whey	Sodium lactate	21	40	15	0.33

Table 3 illustrates the species (T-RFs) that could be identified within the AED culture T-RFLP profiles by comparing them with the T-RFLP of TAN-25 groundwater, which had a portion of the T-RFs identified using clones generated in a clone library (Wood, Cummings, and Sorenson 2002). Using this technique, 14 of the T-RFs within the AED profiles were indirectly identified.

Despite significant differences between the bacterial consortia in the AED cultures, *Dehalococcoides ethenogenes* and *Dehalospirillum multivorans* were both detected within the T-RFLP profiles. These bacteria are of particular importance because they are isolated, characterized, dechlorinating bacteria. *Dehalococcoides ethenogenes,* specifically, is the only known bacterium capable of complete anaerobic reductive dechlorination (ARD) of TCE to ethene. According to the T-RFLP profiles, this bacterium accounted for 27% of the total population in the food-grade molasses culture, 29% in the feed-grade molasses culture, 8% in the cheese whey culture, and 20% in the sodium lactate culture. These data should be interpreted with caution, however, as they were generated using the PCR-amplified community and may not represent the actual relative abundances of members in the community. In general, however, *Dehalococcoides* is under-represented by PCR analysis. The presence of this bacterium within all of the AED cultures indicates that a variety of distinctive cultures can support this dechlorinating microbe.

Table 3. Identified terminal restriction fragments for each of the alternate electron donor cultures[a].

T-KF	Genus of Correlated Clone	Food-Grade Molasses	Feed-Grade Molasses	Cheese Whey	Sodium Lactate
90	*Acetobacterium, Cytophaga*	+	+	+	+
92	*Bacteroides*	+	+	+	+
122	*Spirochete*	+	+	+	+
144	*Cytophaga*	+	+	+	+
163	*Acetobacterium*	+	+	-	+
219	*Acetobacterium*	+	-	-	+
224	*Clostridium, Acetobacterium*	+	-	-	+
262	*Thermospira*	+	+	+	+
278	*Acholeplasma*	-	+	+	+
289	*Syntrophomonas*	+	-	-	+
469	*Dehalospirillum*	+	-	-	-
371	*Syntrophomonas*	+	-	-	+
514	*Dehalococcoides*	+	+	+	+
521	*Clostridium*	+	+	-	+

a. The +/- indicates the presence/absence of the T-RF in the community profile.

Dehalospirillum multivorans is known to dechlorinate TCE to *cis*-DCE, but does not dechlorinate *cis*-DCE or vinyl chloride (VC). Therefore, it may also be important in TCE-dechlorinating systems. According to T-RFLP, however, only the food-grade molasses culture contained this T-RF in the community profile. Certain members of *Acetobacterium* are involved in the anaerobic cometabolism of PCE. These systems, however, are not well understood and cometabolism of other chlorinated compounds may also occur.

The second component in analyzing the T-RFLP data for the AED cultures is the assessment of diversity, which includes both species richness and evenness. The diversity parameter calculations for each of the AED cultures are reported in Table 4. The Shannon-Weiner index range is from 0 to infinite, with 0 indicating one species within the community and a value of 7 to 8 reported for diverse marine environments. The Shannon-Weiner indices for the AED cultures ranged from 4.39 to 5.32, which suggest that these communities are relatively diverse. The Simpson index is the probability that any two individuals chosen at random from a community belong to a different species. It ranges from 0 to 1 with 0 indicating one species within the community and 1 indicating an infinite number of species (no species will ever be chosen twice). The Simpson indices of the AED cultures ranged from 0.75 to 0.91. The difference between the Shannon-Weiner and Simpson indices is that the Simpson places a higher emphasis on the relative proportion between species (evenness) within the community. The Shannon-Weiner function is an indication of evenness of the community and ranges from 0 to 1, with 0 indicating the dominance of one species and 1 indicating all species are equal in proportion. The Shannon-Weiner functions of the AED cultures ranged from 0.66 to 0.79.

The T-RFLP profiles for each AED culture were normalized relative to one another and plotted together on a 3-D graph so that comparisons between the diversity of each culture could be visually assessed (see Figure 1). This figure reveals the relatively high percentage of the total fluorescence that the T-RF for *Dehalococcoides ethenogenes* comprised for each culture. This suggests that this organism is one of the dominant members within the AED cultures.

Table 4. Diversity parameters of alternate electron donor cultures.

Diversity Parameters	Food-Grade Molasses	Feed-Grade Molasses	Cheese Whey	Sodium Lactate
Shannon-Weiner index	4.95	4.46	4.39	5.32
Shannon-Weiner function	0.77	0.66	0.69	0.79
Simpson index	0.87	0.78	0.75	0.91

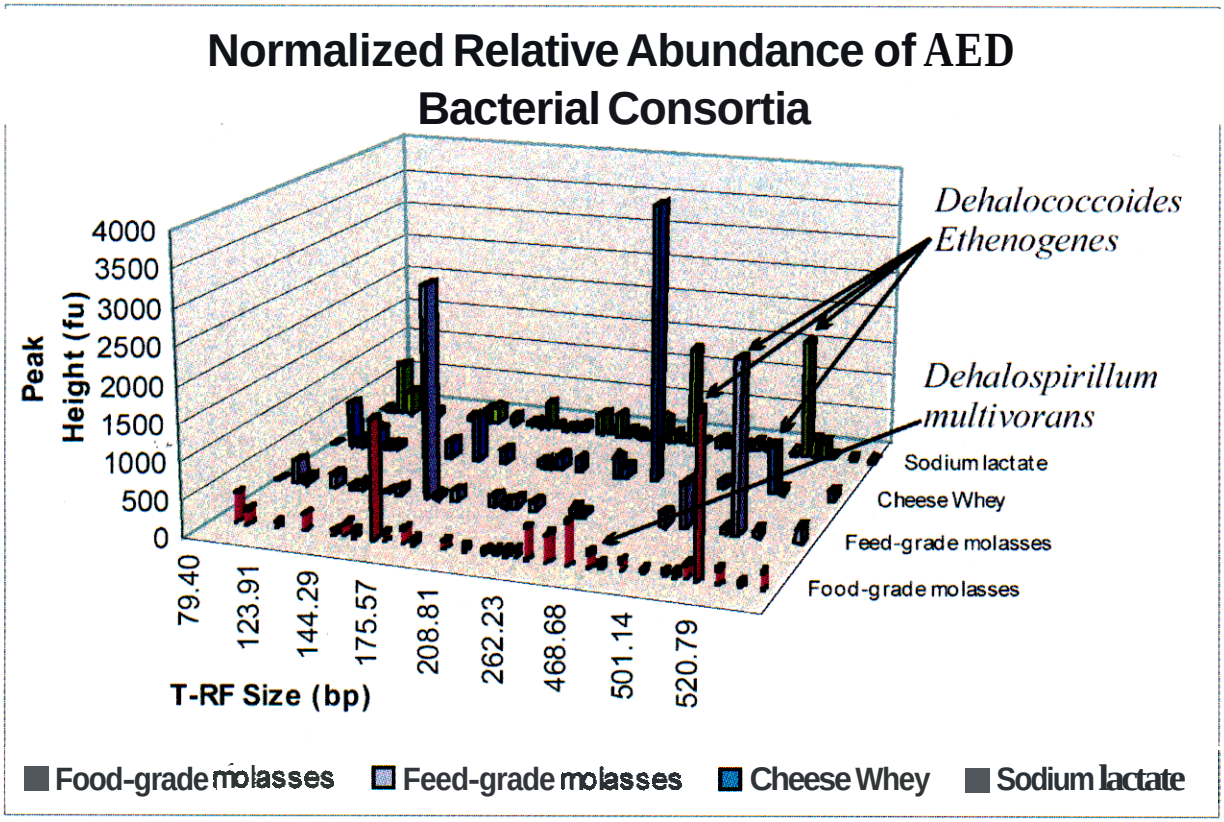

Figure 1. Normalized *Bacteria* terminal restriction fragment length polymorphism profile of alternate electron donor cultures.

3.3 Terminal Restriction Fragment Length Polymorphism Archaea

The results of the archaeal T-RFLP profiles of the AED cultures were very similar (see Figure 2). Each culture's T-RFLP detected three species of *Archaea*. Using clones generated from the archaeal clone library created for TAN-25 groundwater (Wood, Cummings, and Sorenson 2002), two of the three species were identified. They included T-RF 196 bp, which was *Methanothrix*, and T-RF 335, which was *Methanosarcina* and *Methanospirillum*. All of these *Archaea* were methanogens, which is not surprising given that significant methane production was observed in all of the cultures. *Methanothrix* and *Methanosarcina* genus are acetoclastic methanogens and *Methanospirillum* are hydrogenotrophic methanogens.

Methanogens are very important in dechlorinating communities. Hydrogenotrophic methanogens utilize hydrogen, which is also used by most dechlorinating bacteria, including *Dehalococcoides ethenogenes* and *Dehalospirillum multivorans*. Consequently, methanogens compete with dechlorinators for available hydrogen and may actually inhibit dechlorination under certain conditions. Acetoclastic methanogens, on the other hand use acetate and so do not compete with dechlorinators. The predominance of the acetoclastic methanogens within the AED culture T-RFLP profiles (approximately 80% of the total populations) indicates that hydrogenotrophic methanogens may not significantly impact dechlorination in these systems.

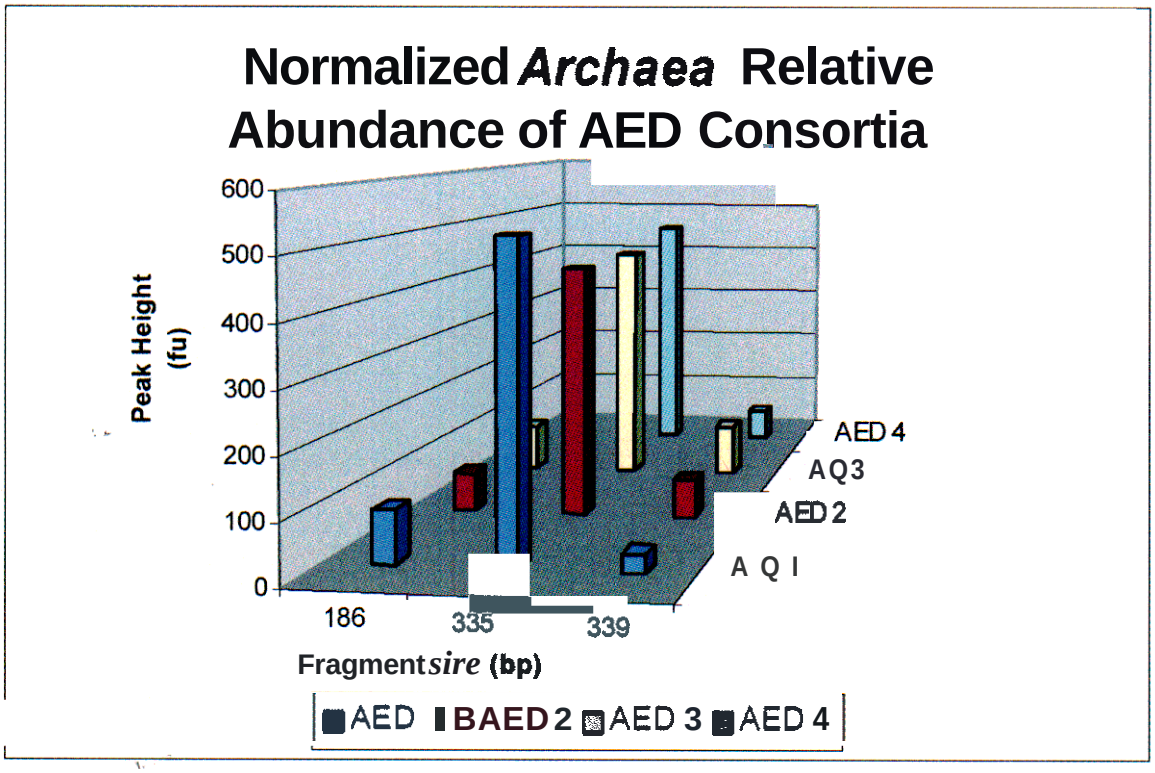

Figure 2. Normalized *Archaea* terminal restriction fragment length polymorphism profile. Food-grade molasses (AED 1), feed-grade molasses (AED 2), cheese whey (AED 3), and sodium lactate (AED 4).

3.4 implications of Microbial Community Structure

The main objective of this study was to determine if the AEDs feed-grade molasses, food-grade molasses, and cheese whey stimulated similar TCE-dechlorinating populations within a microbial community as well or better than sodium lactate. The TCE reduction performances of the AED cultures were significantly different (INEEL 2002). It was hypothesized that these differences in performance may have been due to differences in the community structure, specifically the lack of dechlorinating bacteria within the culture.

According to T-RFLP analysis, all of the communities analyzed were significantly different. More species were found in the lactate culture than in any of the AED cultures (see Table 2). The food-grade molasses culture was more similar to the lactate culture than either the feed-grade molasses or cheese whey cultures (see Table 2). The impurity of the cheese whey and feed-grade molasses may have introduced bacterial populations not originally present within the TAN groundwater. These foreign populations may have contributed to the low TCE-reduction performance observed within these cultures. Consequently, the introduction of foreign populations into the groundwater at TAN may have significant implications to the natural dechlorinating populations. Therefore, the use of electron donors with active cultures should be carefully considered. If the AED does not work in the field, it may be difficult to recover the lactate-derived community because the introduced bacterial populations may out-compete the natural populations.

The differences observed within the communities may also have been due to differences in potential electron donors derived from the parent donors. Feed-grade molasses, food-grade molasses, and cheese whey are all complex electron donors comprised of a variety of chemicals that could potentially be fermented in microbial metabolic reactions. Lactate, conversely, is a simple electron donor whose fermentation pathways have been elucidated clearly. The fermentation of lactate produces acetate, propionate, and hydrogen as secondary electron donors. Thus, the introduction of the AEDs and their fermentation by-products were likely selected for different populations within the communities.

All of these electron donors, however, inevitably produce hydrogen during various anaerobic fermentation reactions. *Dehalococcoides ethenogenes* is an obligate hydrogenotroph and dechlorinator, which may be why this organism was present in all of the AED cultures as one of the dominant species. The prevalence of hydrogen and TCE would have provided strong selective pressure for this organism. Thus, the stimulation of this bacterium may be achieved using a variety of electron donors as long as the fermentations produce hydrogen and the redox conditions allow for dechlorination of the chlorinated ethenes. The presence of this species within the cultures also suggests that the ability to completely dechlorinate TCE was not limited to a reduction or absence of dechlorinating bacteria within the AED cultures.

Other factors that could have influenced the TCE reduction performance within the AED cultures is competition between other microbes and dechlorinating bacteria for limited nutrients, the production of substances that are inhibitory to dechlorination, and/or a mutation of the dechlorinating bacteria so they can no longer dechlorinate one or more of the chlorinated ethenes. These potential effects cannot be elucidated without further study.

3.5 Recommendations

The variation in TCE reduction performance between the AED cultures could not be accounted for by the absence of dechlorinating bacteria. Molecular analyses PCR and T-RFLP suggested that *Dehalococcoides ethenogenes,* which is able to completely dechlorinate TCE to ethene, was present in high proportions in all of the cultures. In addition, *Dehalospirillum multivorans*, which dechlorinates TCE partially to *cis*-DCE, was also present in the food-grade molasses culture.

These data support the conclusion that food-grade molasses and cheese whey are suitable electron donors for stimulating efficient ARD of TCE to ethene. Although dechlorination of sodium lactate was perhaps the most efficient, these other electron donors also support dechlorination (INEEL 2002). This study supports further consideration of cost-benefit analysis and technical feasibility of using these AEDs in the field.

4. SUMMARY AND CONCLUSIONS

Molecular methods were used to evaluate the effect of four different electron donors on the microbial community structure of a TCE-dechlorinating culture. Enrichment cultures derived from TAN groundwater were developed in the laboratory using the electron donors cheese whey, food-grade molasses, feed-grade molasses, and sodium lactate. The TCE reduction performance of these four cultures differed significantly over a 2-year period (INEEL 2002). The differences in reductive performance, however, could not be explained by the absence of dechlorinating bacteria. Molecular analysis T-RFLP indicated significant differences in species composition, but also showed high proportions of dechlorinating bacteria within all the enrichments. The addition of foreign populations within the cheese whey and food-grade molasses, however, may have impacted the dechlorination performance in these cultures. These results illustrate that:

- A variety of electron donors, both in pure form and in complex mixes, can be used successfully to support bacteria capable of complete ARD of TCE to ethene
- Different members of a community are stimulated after an AED has been provided
- Different electron donors selected for distinct populations, and significantly different communities, even though they were derived from the same source
- Foreign populations introduced by impure electron donors may have contributed to impacted dechlorination performance
- *Dehalococcoides ethenogenes* was dominant in all of the cultures; therefore, further evaluation of these electron donors for field application should progress.

5 REFERENCES

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Attachment 1

Alternate Electron Donor Metals Results

Alternate Electron Donor Metals Results

TAL metal	SDWA MCLs, (ug/L)	Allowable injected concentration 10 x MCLs	Stated in TOS 1214 R1 RDLs, (ug/L)	Whey, 7% w/w, filtered ELE10001C0	Whey, 7% w/w, unfiltered ELE10101C1	Sodium dipropionate, 7% w/w, filtered ELE10201C0	Sodium dipropionate, 7% w/w, unfiltered ELE10302C1	Westway feed-grade molasses AEDE0101LA	Westway feed-grade molasses AEDE0101LA	Westway feed-grade molasses AEDE0101LA	Westway feed-grade molasses AEDE0101LA	PM AG Products food-grade molasses AEDE0201LA	International Foods food-grade molasses AEDE0301LA	Groeb Farms food-grade molasses AEDE0401LA	Commerce Light and Sweet molasses AEDE0501LA
Al	NA	NA	200	67.4 B	21.2 U	62.2 B	39.3 B	5.8	207	230	5.8	1.6 U	1.6 U	1.6 U	1.6 U
Sb	6a, b	60	6	5.2 B	5.1 U	5.1 U	5.1 U	0.55	44.3	46.7	0.4	0.43	0.53	0.40 U	0.40 U
As	50c	500	50	2.2 U	2.2 U	2.2 U	2.2 U	0.2	176	185	0.24	0.20 U	0.20 U	0.20 U	0.20 U
Ba	2000a, b	20000	100	2.3 B	1.8 B	5.1 B	3.6 B	9.5	186	202	9.3	0.24	3.1	0.97	1.4
Be	4a, b	40	4	0.30 U	0.52 B	0.30 U	0.30 U	0.10 U	4.6	4.9	0.10 U	0.10 U	0.10 U	0.10 U	0.10 U
Cd	5a, b	50	5	0.78 B	1.2 B	0.85 B	0.78 B	0.10 U	5.5	4.6	0.10 U	0.10 U	0.10 U	0.10 U	0.10 U
Ca	NA	NA	200	34700	36000	372 B	394 B	9700	11200	11500	9630	243	8020	1770	1770
Cr	100a, b	1000	100	5.2	5.0 B	17.3	16.9	0.67	18.5	19.7	0.7	0.24	0.36	0.10 U	0.13
Co	NA	NA	110	1.1 U	1.1 U	3.3 B	1.1 U	1	42.4	45	1.1	0.24	0.20 U	0.20 U	0.20 U
Cu	1300b, d	13000	80	18.5	157	3.6 B	24.4	7.9	31.1	37.7	8	3.6	19.4	0.4	1.22
Fe	NA	NA	1000	27.0 U	62.4 B	588	551	277	352	382	272	83.3	142	53.8	46.4
Pb	15d, 0b	150	15	2.8 U	2.8 U	283	274	0.4	43.3	45.8	0.34	0.51	0.52	0.27	0.23
Mg	NA	NA	200	8630	8650	269	248 B	3850	5940	6010	3790	12.6	2050	955	999
Mn	NA	NA	40	4.9	1.7 B	9.5	10.3	25.3	65.9	69.4	24.9	5.6	5.8	3.5	3.8
Hg	2a, b	20	2	0.10 U	0.10 U	0.10 U	0.10 U	0.031 U	0.17	0.18	0.031 U	0.032 U	0.031 U	0.031 U	0.09
Ni	NA	NA	100	5.5 B	4.9 B	21.7	23.2	2.6	44.2	47.2	2.5	1.2	1.4	0.14	0.44
K	NA	NA	1000	181000	186000	84000	89700	31700	32100	32800	30900	26600	21800	8520	71900
Se	50a, b	500	50	4.7 B	6.9 B	32.3	28.5	3.2	185	194	3.2	1.3	1.1	1.1	1.5
Ag	NA	NA	10	1.8 U	3.3 B	3.0 B	1.8 U	0.20 U	4.4	4.4	0.20 U	0.20 U	0.20 U	0.20 U	0.20 U
Na	NA	NA	1000	49400	49500	12700000	13400000	1350	3130	3310	1360	11000	1260	292	462
Th	2a, 0.5b	20	2	4.1 U	4.1 U	4.1 U	4.1 U								
V	NA	NA	100	0.80 U	0.80 U	1.6 B	0.80 U	1.4	44	46.8	1.4	0.12	0.29	0.10 U	0.10 U
Zn	NA	NA	100	14.6 B	21.6	3410	3550	10.3	49.1	53.3	7.8	15.7	13	2.5	3.5

a = 40 CFR 141.62
b = 40 CFR 141.55, MCLGs
c = 40 CFR 141.11
d = 40 CFR 141.80

Date qualifiers:
U = Analyte was analyzed for but not detected.
B = Value is less than the CRDL but greater than the IDL.
Highlighted values equal or exceed allowable injection concentrations

Appendix F

Quality Assurance Details

Appendix F

Quality Assurance Details

I. IN SITU BIOREMEDIATION DATA QUALITY ASSURANCE

General quality assurance (QA) requirements for Idaho National Engineering and Environmental Laboratory (INEEL) Environmental Restoration (ER) projects are established in the ER *Quality Assurance Project Plan for Waste Area Groups 1, 2, 3, 4, 5, 6, 7, 10, and Inactive Sites (DOE-ID* 2002) and the *Project Management Plan, Environmental Restoration Program Management* (INEEL 2000). Specific precision and accuracy requirements defined in the *Sampling and Analysis Plan for Enhanced In Situ Bioremediation Predesign Operations Test Area North (INEEL* 2001) are summarized in Table 1. Duplicates, field blanks, and trip blanks are used, as specified in the Quality Assurance Project Plan (QAPjP; DOE-ID 2002). Results for accuracy, precision, and completeness are provided in this appendix.

Although Table 1 specifies the precision and accuracy requirements stated in the Predesign Operations (PDO) Sampling and Analysis Plan *(SAP;* INEEL 2001), more stringent QA measures were developed in the *In Situ Bioremediation Remedial Action Groundwater Monitoring Plan for Test Area North, Operable Unit 1-07B* (INEEL 2002) and ancillary documents. Several of these more stringent measures were implemented for data analyzed in the in situ bioremediation (ISB) field laboratory starting in April 2002. These measures are specified in the latest version of TPR-166, "In Situ Bioremediation Field Laboratory Procedure."

Table 1. Precision and accuracy requirements for Operable Unit 1-07B in situ bioremediation performance monitoring from the Predesign Operations Sampling and Analysis Plan (INEEL 2001).

Analyte	Potential Analytical Method	Precision/Accuracy (%)
Chloroethenes	GC/ECD	± 25
Ethene/ethane/methane	GC/FID	± 25
Lactate	IC	± 25
Acetate/propionate/butyrate	GC/FID	± 50
Sulfate	Hach field test kit	± 50
COD	Mach field test kit	± 50
Tritium	Liquid scintillation counting	± 25
Iron	Hach field test kit	± 50
Alkalinity	Hach field test kit	± 25
Sr-90/Cs-137/alpha emitters	Gas flow proportional gamma spectrometry	Not specified
Metals	SW-846, 6010B, and 7000 series	Not specified

GC/ECD = gas chromatography/electron capture detector
GC/FID = gas chromatography/flame ionization detection
IC = ion chromatography

According to the ISB PDO SAP (INEEL 2001), all of the data collected during the PDO period were to be used to evaluate relatively long-term changes in analytes; thus, no single sample was critical to the interpretation. The quality level defined for all sampling activities in this plan was screening data, in accordance with the data quality objective (DQO) process (EPA 1993) and the QAPjP (DOE-ID 2002); however, the SAP stated that most of the quality assurance/quality control (QA/QC) elements required for definitive data were to be used. The SAP further stated that definitive confirmation was to be provided for the chloroethene data and the ethene/ethane/methane data by sending splits to an off-site laboratory on a quarterly basis. Definitive data underwent Level A validation by the INEEL Sample and Management Office (SMO); all other data from off-site laboratories received completeness and QC checks.

1.1 Accuracy

Accuracy is a measure of bias in the sampling and analysis program. It can be affected by the methods used for sampling preservation and handling, by the sample matrix, and by analytical methods. During this reporting period, accuracy was assessed through analysis of standards, standard additions, splits, performance evaluation (PE) samples, blanks, and matrix spike and matrix spike duplicate (MS/MSD) data.

Standards—Standards were used to determine the accuracy of analyses conducted in the ISB field laboratory, including COD, sulfate, iron, phosphate, and ammonia. A COD standard was analyzed with each set of COD samples during this reporting period. Starting in April 2002, standards for sulfate, iron, phosphate, and ammonia were analyzed each day the analyses were conducted. Table 2 presents accuracy results for standards, including the type of analyte, a count of number of times a standard was performed during this reporting period, averages of the percent recovery values, the standard deviation of all percent recoveries from the reporting period, averages of the percent error values, and whether the PDO SAP criteria (Table 1) was met, as determined from the percent error value. Percent recovery was calculated as:

$$\%\ Recovery = \frac{Observed\ Value}{Standard\ Value} x\ 100\% \qquad (1)$$

where

Observed Value = result of analysis

Standard Value = value of standard solution.

Percent error was calculated as:

$$\%\ Error = \left| \frac{Observed\ Value - Standard\ Value}{Observed\ Value} \right| x\ 100\%. \qquad (2)$$

Standards data are reported in Appendix D. Accuracy requirements presented in Table 1 were met for each of these parameters.

Analyte	Count	Average Percent Recovery	Percent Recovery Standard Deviation	Average Percent Error	PDO SAP Criteria Met?
COD	24	98.0	8.40	7.0	Yes
Sulfate	29	117	9.86	15	Yes
Iron	17	86.1	17.2	24	Yes
Phosphate	6	110	9.03	8.2	Yes
Ammonia	3	106	4.93	6	Yes

Table 3. Accuracy of in situ bioremediation field laboratory standard additions.

Analyte	Count	Average Percent Recovery	Percent Recovery Standard Deviation	Average Percent Error	PDO SAP Criteria Met?
Alkalinity	8	100.7	5.99	10	Yes
Sulfate	3	70.2	55.1	4s	Yes
Phosphate	1	138.8	N/A	39	N/A
Ammonia	1	98.0	N/A	2	N/A

Splits—On a quarterly basis during this reporting period, split samples for chloroethenes and ethene/ethane/methane samples from all ISB wells were sent both to the INEEL Research Center (TRC) and to an off-site laboratory. The off-site laboratories used EPA Method 8260B (EPA 1996) for independent verification of the IRC solid-phase microextraction (SPME) results. The target relative percent difference (RPD) for splits identified in the PDO *SAP* (INEEL 2001) was 25%.

The RPD for split samples (DOE-ID 2002) is calculated as:

$$RPD = \frac{|C_1 - C_2|}{\left[\frac{C_1 + C_2}{2}\right]} \times 100\% \qquad (3)$$

where

C_1, C_2 = analyte concentrations determined for duplicate or split samples.

Tables 4 and 5 present the RPD averages and standard deviations for chloroethenes and ethene/ethane/methane, in addition to numbers and percentage of split analyses less than 25 and 50% RPD for each parameter. RPDs were not calculated when IRC results were reported as not detected or trace. RPDs were calculated using off-site laboratory results that had been flagged as an estimated or undetected value.

The target RFD of 25% was only met for trans-DCE. For some parameters, high RPD values can be partially attributed to calculating the RPD between two low values (<10 ppb). Since split accuracy between the IRC and off-site laboratories was not within the target RPD range, performance evaluation (PE) samples and duplicate samples were examined for both laboratories to determine individual accuracy and precision at both the on-site and off-site laboratories.

***Performance Evaluation* Samples—Performance** evaluation samples were analyzed for chloroethenes using the SPME method at the IRC and the EPA 8260B method at an off-site laboratory. During this reporting period, the off-site laboratory was Severn Trent Laboratories. The PE program was administered by the INEEL SMO using commercially supplied certified standards. The PDO SAP (INEEL 2001) describes the details of this program. PE samples were purchased and prepared by Environmental Resource Associates and shipped directly from this vendor to the INEEL ISB Field Team. Field team members included the PE samples with the other ISB samples collected during that sampling event, which were all sent together to the IRC and Severn Trent Laboratories.

During this reporting period, PE samples were sent monthly to the IRC from February 2002 to September 2002 for both high (>100 ppb) and low (<100 ppb) range chloroethene concentrations. Since chloroethenes were only sent to an off-site laboratory on a quarterly basis, two PE samples (one low range [February 2002] and one high range [May 20021) were sent to Severn Trent Laboratories for analysis during this reporting period.

Performance evaluation results for the SPME method are presented in Table 6. EPA 8260B results are presented in Table 7. For all PE samples collected, the average and standard deviation of the percent recovery are shown for all data points, including duplicate values as individual data points. The number and percent of values within accuracy limits shown represent data points that fell within the 9.5% confidence limits established by Environmental Resource Associates. SPME results showed high numbers of values falling within the accuracy limits for all parameters except low range PCE and low range vinyl chloride (VC). EPA 8260B results were limited by only two reported data points for all parameters. Low range results for all parameters, collected in February 2002, fell within the accuracy limits, whereas high range results for all parameters collected in May 2002 were significantly lower than the certified value. For example, the certified value for PCE was 322 µg/L, but the EPA 8260B result was reported as 1.4 µg/L. High range inaccuracies could have resulted from improper sample preparation, improper storage of the sample, or problems during analysis.

The number of duplicates, average RPD, and RPD standard deviation values are shown for duplicate PE samples sent to the IRC (Table 6). No duplicate PE samples were sent to Severn Trent Laboratories. The number and percent of values within precision limits represent the number of duplicate data points with RPDs below 25%. All SPME reported values fell within the precision limits.

loroethenes split analyses.

	TCE			*trans-DCE*			*cis-DCE*			*PCE*			*VC*		
Statistic	*All data*	*<100 ppb*	*>100 ppb*	*All data*	*<100 ppb*	*>100 ppb*	*All data*	*<100 ppb*	*>100 ppb*	*All data*	*<100 ppb*	*>100 ppb*	*All data*	*<100 ppb*	*>100 ppb*
count	**63**	43	20	**63**	**28**	*35*	**63**	**46**	**17**	*32*	*32*	N/A	**49**	**45**	**4**
Average RPD	**43.9**	**52.7**	**25.0**	**12.8**	**12.0**	**13.5**	**65.8**	*96.6*	**36.7**	55.4	55.4	N/A	63.6	**59.6**	**109.2**
RPD **Std.** Deviation	**44.6**	**50.2**	19.5	**18.6**	21.2	**16.4**	**55.6**	**54.8**	**48.1**	39.3	39.3	N/A	**43.4**	42.1	**32.6**
Count<25% RPD	31	18	**13**	**55**	**25**	**30**	**20**	10	10	12	**12**	N/A	**12**	**12**	**0**
%<25% RPD	**49.2**	**41.9**	*65.0*	87.3	**89.3**	**85.7**	31.8	**21.7**	**58.8**	**37.5**	37.5	N/A	**20**	**26.7**	*0*
Count<50% RPD	**45**	**27**	18	**59**	**27**	32	**43**	**17**	**14**	**20**	**20**	**N/A**	20	*20*	*0*
%<50% RPD	**71.4**	**62.8**	90.0	93.7	**96.4**	91.4	**68.3**	**37.0**	**82.4**	**62.5**	**62.5**	**N/A**	**41**	44.4	0

Table 5. Relative percent differences for ethene/ethane/methane **split analyses.**

	Ethane			***Ethene***			*Methane*		
Statistic	*All data*	*<100 ppb*	*>100 ppb*	***All datu***	*<100 ppb*	*>100 ppb*	*All data*	*<100 ppb*	*>100 ppb*
count	**8**	**8**	**NIA**	**46**	**36**	10	**71**	*5*	**66**
Average RPD	**50.5**	**50.5**	N/A	**29.7**	**31.7**	*22.6*	**58.4**	**117.8**	**53.9**
RPD Std. Deviation	**54.65**	**54.65**	**N/A**	**25.94**	*26.23*	**24.82**	**46.37**	**48.18**	*43.38*
Count <25% RPD	**4**	**4**	N/A	*23*	**17**	**6**	**19**	0	**19**
% <25% RPD	**50**	**50**	N/A	**50**	**47.22**	60	**26.76**	0	**28.79**
Count <50% RPD	**6**	*6*	**N/A**	**37**	**28**	**9**	**40**	1	**39**
% <50% RPD	**75**	**75**	N/A	**80.43**	**77.78**	90	56.33	20	**59.09**

Table 6. SPME[1] performance evaluation sample results.

Statistic	TCE			trans-DCE			cis-DCE			PCE			VC		
	All data	<100 ppb	>100 ppb	All data	<100 ppb	>100 ppb	All data	<100 ppb	>100 ppb	All data	<100 ppb	>100 ppb	All data	<100 ppb	>100 ppb
Count	24	10	14	24	10	14	24	10	14	24	10	14	24	10	14
Average % Recovery	87.2	83.8	89.6	97.6	92.9	101	100	92.5	105	74.4	64.7	81.3	106	88.8	119
% Recovery Std. Deviation	17.8	13.3	20.6	18.3	16.1	19.6	20.6	17.7	21.4	24.9	30.4	18.3	34.0	28.6	32.8
Count within accuracy limits	20	9	11	22	10	12	22	10	12	17	4	13	11	0	11
% within accuracy limits	83.3	90	78.6	91.7	100	85.7	91.7	100	85.7	70.8	40	92.9	45.8	0	78.6
Count duplicates	11	4	7	11	4	7	11	4	7	11	4	7	11	4	7
Average RPD	2.57	2.42	2.66	1.73	1.76	1.72	1.11	1.08	1.13	3.29	0.67	4.78	2.92	1.06	3.98
RPD Std. Deviation	2.36	2.01	2.69	2.04	2.73	1.78	0.94	1.13	0.91	4.39	0.82	4.97	2.95	1.66	3.09
Count within precision limits	11	4	7	11	4	7	11	4	7	11	4	7	11	4	3
% within precision limits	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100

1 = Analyses performed at INEEL IRC

Table 7. EPA 8260B[1] performance evaluation sample results.

Statistic	TCE			trans-DCE			cis-DCE			PCE			VC		
	All data	<100 ppb	>100 ppb			>100	All data	<100 ppb	>100 ppb	All data	<100 ppb	>100 pph	All data	<100 ppb	>100 ppb
count	2	1	1				2	1	1	2	1	1	2	1	1
Average % Recovery	70.2	77.4	63.1				53.2	98.8	7.6	34.9	69.4	0.43	23.6	41.7	5.5
% Recovery Std. Deviation	10.1	NA	NA				64.5	NA	NA	48.7	NA	NA	25.6	NA	NA
Count within accuracy limits	1	1	0				1	1	0	1	1	0	1	1	0
% within accuracy limits	50	100	0	50	100	0	50	100	0	50	100	0	50	100	0

1 = Analyses performed at Severn Trent Laboratories.

Blanks — The PDO **SAP** requirements included collecting one trip blank per sampling event for chloroethenes and ethene/ethane/methane analyses and collecting field blanks at a frequency of one per sampling event for radiological analyses. **Future** QA goals, stated in the ISB Groundwater Monitoring Plan {INEEL2002), include collecting one **trip blank** per sample cooler **for** chloroethenes and ethene/ethane/methane **and** collecting one field **blank** per 20 samples for all samples (one **sample** for all analytes per day **if** the number of monitoring locations is <20).

During the reporting period, the PDO *SAP* requirements were met and additional blanks were collected to implement future **QA** goals. **At least** one trip blank was collected for chloroethenes and ethene/ethane/methane for each sampling event; for all sampling events except three, one **trip** blank was collected **per** sample cooler. Field blanks were collected **at a** frequency of one per sampling event for **all** radiological analyses and were collected **for** chloroethenes during 13 **sampling** events and for ethene/ethane/methane during **12** sampling events. **For the trip and** field blanks collected during this reporting period, there were no false positive values with **a** single exception; the tritium field blank collected on **July** 9,2002 reported a value of 2,000 pCi/L.

Analyte	Count	Average Percent Recovery	Percent Recovery Standard Deviation	Future Criteria Met?
PCE	6	94.2	3.38	Yes
TCE	6	102	4.46	Yes
cis-DCE	6	104	5.45	*Yes*
trans-DCE	6	102	2.06	Yes
VC	***6***	**114**	10.1	**Yes**

Table *9.* Off-site laboratow matrix spike/matrix spike duplicate data.

Analyte	Count	Average Percent Recovery	Percent Recovery Standard Deviation	Future Criteria Met?
TCE	8	95.2	23.5	**Yes**
Ethane	10	**50.2**	52.1	Yes
Ethene	10	41.3	50.6	**Yes**
Methane	10	-1,295	2,827	**NO**

I.2 Precision

Precision is an assessment **of** reproducibility of measurements under a given set **of** conditions. Overall precision was assessed through collection and analysis **of** duplicate samples at the **TSB** field laboratory, lRC, and off-site laboratories. Duplicate samples are defined as two samples collected **for** the same analyses during **a** single mobilization. Average RPD and RPD standard deviations are presented for all duplicate data **in** Tables 10 through **14.**

Analyte	Count	Average RPD	RPD Standard Deviation	Count <25% RPD	Count <50% RPD
Alkalinity	16	1.55	1.03	16	16
Ammonia	2	4.00	N/A	2	2
Phosphate	2	6.50	NIA	2	2
Iron	2	16.4	N/A	1	2
Sulfate	11	3.64	2.31	11	11
COD	4	31.2	37.3	2	3

Table 11. Relative percent differences for INEEL Research Center duplicates.

Analyte	Count	Average RPD	RPD Standard Deviation	Count <25% RPD	Count <50% RPD
PCE	2	14.9	N/A	2	2
TCE	11	2.64	1.53	11	11
cis-DCE	5	4.09	3.00	5	5
trans-DCE	8	3.45	2.27	8	8
VC	2	9.95	N/A	2	2
Ethene	7	26.7	21.6	4	6
Methane	15	19.4	17.4	10	14
Lactate	2	0.59	N/A	2	2
Propionate	2	3.47	NIA	2	2
Acetate	3	2.82	2.24	3	3
Butyrate	2	5.24	N/A	2	2

Analyte	Count	Average RPD	RFD Standard Deviation	Count <25% RPD	Count <50% RPD
PCE	2	63.8	N/A	1	1
TCE	5	6.9	4.85	5	5
cis-DCE	4	5.4	2.46	4	4
trans-DCE	5	5.9	2.48	5	5
VC	4	36.9	31.3	2	3
'Ethene	4	17.3	10.4	3	4
Ethane	2	11.9	N/A	2	2
Methane	5	75.1	62.5	2	2

Analyte	Count	Average RPD	RPD Standard Deviation	Count <25% RPD	Count <50% RPD
Tritium	15	8.60	9.33	13	I5
Sr-90	5	3.07	2.40	5	5
Cs-137[2]	2	5.33	N/A	2	2

Analyte	Count	Average RPD	RPD Standard Deviation	Count <25% RPD	Count <50% RPD
Barium	5	4.88	5.04	5	5
Calcium	5	4.65	4.42	5	5
Chromium	1	14.6	N/A	1	1
Iron	4	5.24	4.68	4	4
Magnesium	5	5.42	4.80	5	5
Manganese	4	5.45	3.41	3	4
Nickel	1	2.51	N/A	1	1
Potassium	5	4.70	4.08	5	5
Sodium	5	3.93	2.91	5	5
Zinc	4	10.0	10.2	4	4

1. All analyses performed at Southwest Laboratory of Oklahoma.

As shown in Table 10, the average RPD values for all analyses conducted at the ISB field laboratory were within the criteria established in Table 1. Table I 1 shows that the average RPDs for IRC duplicates were within the 25% criteria defined in Table 1 with the exception of ethene, which had an average RPD of 26.7%, only slightly outside the specified range.

All off-site chloroethenes and ethene/ethane/methane duplicates were within the precision criteria except PCE, VC, and methane. Both PCE and VC were present at relatively low concentrations and were usually flagged as an estimated or undetected value; therefore, it is difficult to produce low RPD values. For methane, the off-site reported duplicate values were not similar (910 and 12,000 ppb), which resulted in high RPDs. Average RPDs for radionuclides and metals (Tables 13 and 14) met the criteria presented in Table 1.

1.3 Completeness

Completeness is calculated by comparing the number of samples planned (as listed in the SAP table for each sampling event) to the number of planned samples actually collected, as shown in the following equation:

$$\%C = \frac{Sn}{St} x100\% \qquad (4)$$

where

%C = percent completeness

S_n = number of planned samples collected

S_t = number of samples planned in the SAP table.

The completeness goal, as stated in the PDO SAP (INEEL 2001), was 90%. Completeness results are presented in Table 4-14 of this report. This table shows the number of samples planned (as listed in the SAP table), the number of those planned samples collected, and percent completeness for those samples. As is shown in Table 4-14, a percent completeness of 96.9% was achieved for the reporting period. Additional samples were collected for five sampling events during this reporting period that did not appear on the SAP table for that sampling event. These samples were added to the total planned samples collected and shown in the total samples collected column. Details are provided in the comments column.

2. TRACER TEST QUALITY ASSURANCE

This section presents the results of the QA/QC elements that were employed during the 2002 Tracer Test. These included analyzing standards, blanks, and duplicates. The sample completeness goal for the 2002 Tracer Test was 90% for bromide, iodide, and COD samples, with duplicates being collected at a frequency of 1 per 20 samples (5%). Table 15 presents the completeness percentages and duplicate frequencies. As indicated in Table 15, the goal of 90% completeness was met and duplicates were taken at a frequency of at least 1 per 20 samples.

Table 15. Completeness and duplicate collection frequency for the 2002 Tracer Test.

Analyte	Planned Samples	Actual Samples Collected	Duplicates Collected	Percent Completeness (%)	Duplicate Frequency
Day 1 – Bromide	137	136	7	99.3	1 per 19.6 samples
Day 2 – Iodide/COD	165	156	9	94.5	1 per 18.3 samples
Day 3-11 – Iodide/COD	82	81	7	98.8	1 per 11.7 samples
Total	384	373	23	97.1	1 per 16.7 samples

Accuracy is a measure of bias in the sampling and analysis program. Accuracy can be affected by the methods used for sampling preservation and handling, by the sample matrix, and by analytical methods. For the 2002 Tracer Test, bromide and iodide concentrations were analyzed using Orion™ ion-specific electrodes (ISEs), and COD was analyzed using Hach Method 10067 (a field laboratory analysis). The accuracy goal for bromide, iodide, and COD was an average percent recovery between 90 and 110%. To ensure accuracy for the bromide and iodide analyses, the bromide and iodide ISE readings were compared with standard solutions of 1, 10, 100, and 1,000 mg/L concentrations at a frequency of at least 1 per 20 samples. If the ISE gave a reading outside the 90 to 110% range, it was standardized according to the equipment manufacturer's procedure. Due to the high frequency of comparing ISE readings with the standard solutions, matrix spikes were not analyzed for bromide or iodide. To ensure accuracy for the COD analyses, each COD batch analyzed included an 800-mg/L standard. Table 16 shows that the percent recovery values based on this standard were all within the target of 90 to 110%.

Precision is an assessment of reproducibility of measurements under a given set of conditions. Overall precision can be assessed through the collection of duplicate samples, which are defined as two samples collected for the same analysis during a single mobilization. As discussed earlier, duplicates were collected at a frequency of at least 1 duplicate per 20 samples (Table 15) during the 2002 Tracer Test. Target RPD values for bromide and iodide were set at 10%. Target RPD values for COD were set at 50% for samples with a COD concentration less than or equal to 125 mg/L and 25% for samples with COD concentrations greater than 125 mg/L. Table 17 shows the actual RPDs between duplicate samples for bromide and iodide, and Table 18 presents the actual RPDs between duplicate samples for COD.

Table 16. Percent recoveries for 2002 Tracer Test 800-mg/L chemical oxygen demand standard.

Batch Number	Reading (mg/L)	% Recovery
1	868	109
2	813	102
3*	794	99.3
4	799	100
5	877	110
6	791	98.9
7	871	109
8	845	106
9	770	96.3
10	840	105
11	850	106
12	840	105
13	771	96.4
14	729	91.1
15	842	105
Average	NA	103

* The original reading was 902 mg/L; however, the batch was reanalyzed resulting in the new standard reading of 794 mg/L

Table 17. Relative **percent differences** between duplicate bromide and iodide **samples** from the 2002 **Tracer Test.**

Analyte	Concentration (ppm)	RPD (%)
Bromide	2.6/1.46	56*
Bromide	1,030/1,020	1
Bromide	2981286	4
Bromide	1481164	10
Bromide	19.4/38.2	65*
Bromide	266/262	2
Bromide	142/154	8
Iodide	60.8/64.8	6
Iodide	1,260/1,250	1
Iodide	372.51372.5	0
Iodide	234/219	7
Iodide	158/157	1
Iodide	0.295/0.315	a
Iodide	1,150/1,070	7
Iodide	293/288	2
Iodide	228/241	6
Iodide	69.1/70.7	2
Iodide	0.0341/0.0332	3
Iodide	2.04/1.96	4
Iodide	0.076/0.066	14*
Iodide	0.61410.471	26*
Bromide	2.611.46	56*

* Values exceed the target RPD for duplicates.

Table 18. Relative percent differences between duplicate chemical oxygen demand samples from the 2002 Tracer Test.

Concentration (mg/L)	RPD
459/492	7
16,740/15,300	9
21,816/23,868	9
25,416/19,692	25
25,272/21,744	15
240/252	5
24,624/24,480	1
15,408/1,044	175*
15,372/11,160	32*
719	25
90/88	2
15/22	38"
23/10	26
52 02/4563	13
47/25	61*

* Values exceed the target RPD for duplicates.

The RPD values for bromide and iodide duplicates were within the target 10% for all analyses except for two bromide and two iodide duplicates. Of the two bromide duplicates, one had a low concentration (<2.6 ppm), which is close to the detection limit using direct measurement with the Orion™ ISE. The remaining bromide duplicate (19.4 and 38.2 ppm) had a high RPD of 65.3%; however, it appears that the problem was limited to this duplicate sample since all other duplicates were well within the acceptable range. In addition, this duplicate value did not alter the data trend that created the bromide tracer breakthrough curve. Both iodide duplicates had very low concentrations (<0.614 ppm), which resulted in apparently high RPD values, while the actual differences were only 0.01 and 0.14 ppm.

All but *three* of the COD duplicates met the target RPD values of 50% for values less than or equal to 125 mg/L and 25% for values greater than 125 mg/L. Of these, the 175% RPD likely represents a dilution error. While the other two sets of duplicates had RPDs that exceeded the target range, this did not compromise the ability to interpret the results of the tracer test.

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